

1 Targeted next generation sequencing (NGS) of the *CFTR* locus: Comparison of three technical approaches

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Twenty-three years after the discovery of the *CFTR* gene, more than 1900 anomalies are described in the world, the majority of them being single base-pair substitutions or micro-insertions/deletions. Identification of mutations has important implications for genetic counselling, prenatal diagnosis, cascade screening in families, as well as for understanding the genotype–phenotype relationship.

Since a few years, NGS technologies enable us to overcome the classical approaches of whole coding sequence sequencing at single nucleotide resolution.

The aim of our study is to compare 3 different strategies with the Ion Torrent technology (Life Technologies, LT): Ampliseq (LT), Haloplex (Agilent) and home-made Long Range PCR (LR).

Preliminary results showed that the coverage of the *CFTR* locus by Ampliseq is limited to 51% and includes 86% of exons with a depth generally consistent between amplicons. Haloplex approach gives more heterogeneous depth and coverage.

Finally, LR-PCR showed coverage consistent with the design but differences at depth between each LR amplification.

The development of libraries by the two first approaches is rapid (less than 24 h) and technically easy. For LR-PCR, it takes about two days to build a library with long and time-consuming manual steps.

Sequencing result for the *CFTR* gene were validated with wild-type samples and DNA carrying known variants (mutations and polymorphisms) previously identified by DHPLC, HRM and sequencing.

In conclusion, the sequencing of the entire *CFTR* locus by NGS is a tool that can quickly respond to issues such as prenatal diagnostic and the most promising approach seems to be the LR strategy.

2 New insights in CF molecular diagnostics applying the next generation sequencing technologies

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Overall, multiplex analysis of common CF mutations facilitates the CF molecular diagnostics; however, despite the availability of different commercial panels, the high allelic heterogeneity of the *CFTR* gene (near 2000 mutations) makes still difficult the characterization of CF mutations, especially in those populations with a wide mutational spectrum. Next Generation Sequencing (NGS) technologies have proven a high sensitivity to detect sequence variants, irrespectively of their frequency. Herein, we have assessed the suitability of this technology to be applied in CF molecular diagnostics using a cohort of 30 CF patients well characterized. In total, 48 mutations along the gene have been selected for this blind study, considering common and rare mutations mainly, missense (n=23), nonsense (n=8), in/dels (n=2), frameshift (n=4) and splicing (n=11) mutations. Furthermore, the cohort includes heterozygous and homozygous samples as well as some complex alleles. Sequence capture was performed using the NimbleGen sequence capture technology. On average, >99% of all targeted bases were covered by at least 5 reads with a mean coverage achieved of 231X. Thus, the NGS sequencing data provided an adequate depth of sequencing coverage to accurately detect the *CFTR* exome variants. In fact, all CF mutations and different coding SNPs were successfully identified. In conclusion, this pilot study supports that NGS technologies provide precise and quick results and are ready to be implemented in routine genetic diagnostics.

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3 Evaluation of the Multiplicom *CFTR* MASTR V2 kit on a Roche 454 Junior NGS platform as a diagnostic tool for a comprehensive analysis of the *CFTR* gene

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Objectives: Molecular diagnosis of cystic fibrosis and *CFTR*-RD led to the worldwide identification of nearly 2000 sequence variations in the *CFTR* gene.

To improve efficiency of current scanning methods, we evaluated in two French laboratories the *CFTR* MASTR V2 kit designed to allow in a single condition reaction the detection of point mutations, microinsertions/deletions in the 27 coding and flanking sequences, plus 3 deep intronic mutations and Copy Number Variations (CNVs).

Methods: 39 samples heterozygous or compound heterozygous for 39 different point mutations and 5 CNVs previously characterized by Sanger sequencing, QMPSP or MLPA were re-analyzed on the Roche Junior NGS. The sizes of amplicons were controlled by Genescan Analysis on an ABI platform using GeneMapper software. Data was analyzed using AVA (Roche) and/or SeqNext softwares (JSI Medical System).

On the 39 mutations, 33 were correctly identified; 3, located in exonic homopolymer stretches, were indistinguishable by the software but were detected by GeneScan on the basis of abnormal amplicon sizes; 3 were TGmTn mutations (intron 9) and showed a correctly identified T5 repeat and an inexact TG repeat. Large deletions were all identified but a high rate of false positives was observed; large duplications were not tested.

Conclusion: This technique enables a rapid and high-performance analysis for the determination of mutations. The results confirm that homopolymer stretches are the main limitation of pyrosequencing technologies and underline the importance of the GeneScan control to limit the risk of false negatives in these regions. CNVs detection requires optimization and further experiments.

4 Multiplex amplification of specific targets for resequencing using next generation sequencing technology, applied in molecular diagnostics of cystic fibrosis

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Objectives: Next generation sequencing (NGS) is a revolutionary technology that delivers fast, inexpensive and accurate genome information. NGS method allows the parallel sequencing of multiple DNA fragments. The aim of this work was to implement the combination of multiplex amplification assay with contemporary massive parallel sequencing technology for molecular diagnostic application.

Methods: We evaluated Multiplex Amplification of Specific Targets for Resequencing (MASTR™, Multiplicon) assay for the analysis of the *CFTR* gene on the Roche 454 GS Junior system sequencer, to identify disease-causing mutations in cystic fibrosis (CF) patients. CF patient samples were analysed using the *CFTR* MASTR assay amplified 35 amplicons (330–490 bp) of coding region of the *CFTR* gene. The sequencing data were analysed with the software Sequence Pilot (SeqNext module, JSI Medical Systems). A first evaluation relied on the analysis of DNA templates containing known variation to generate a control sample. Secondly, we analysed CF patient samples with unknown CF pathological genotype. In the initial evaluation, all known heterozygous mutations were detected. The percentage of mutant reads ranged from 40% to 60%. Special attention was given to sequencing accuracy in homopolymers. We were able to reliably distinguish mutations from noise through the analysis of the raw signal intensities in homopolymers.

Conclusion: This work presents an evaluation of NGS for use in diagnostics. We anticipate that the technique would further improve, and would allow reducing the costs per analysis and the turn-around time, to benefit patients who undergo *CFTR* molecular testing.